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Stabilized coenzyme solutions and their use for determining dehydrogenases or substrates thereof

The invention concerns stabilized aqueous solutions of a coenzyme for hydrogen-transferring enzymes and their use for determining a corresponding analyte (substrate) in a reduced form or for determining the enzyme activity of a corresponding dehydrogenase. The stabilized solution contains an organic compound or appropriate salts having a pKa value between 1.5 and 6.0 and/or a hydroxylamine derivative.

The determination of enzyme activities (or substrate concentrations), especially in blood serum or plasma, plays an important role in clinical chemical diagnostics. Test procedures are often used for this which are based on the reduction of nicotinamide adenine dinucleotide ("NAD") or nicotinamide adenine dinucleotide phosphate ("NADP") and photometric detection of the resulting change of the absorption behaviour in the ultraviolet wavelength range (λ=334, 340 or 365 nm). When suitable test conditions have been selected, this change is linearly proportional to the enzyme activity (or substrate concentration) to be determined.

Nowadays the methods described in Eur. J. Clin. Chem. Clin. Biochem. 31, 897 (1994) and Eur. J. Clin. Chem. Clin. Biochem. 32, 639 (1994) are generally recommended for determining the enzyme activity of for example lactate dehydrogenase (LDH, E.C.1.1.1.27). The test

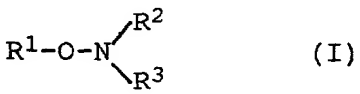
principle involves the oxidation of lactate to pyruvate while a coenzyme such as NAD or NADP is simultaneously reduced to NADH or NADPH. Such a conversion, in this case is for example catalysed by LDH, takes place in an alkaline medium (pH 9.4). As a result of this instability there is a relatively rapid increase in absorbance (the so-called reagent blank) in the wavelength range for the measurement and hence the reagent combination becomes unusable already after a short time (3 months) even when stored in a refrigerator (2° to 8°C). This is a particular problem for the production of ready-to-use liquid reagents with a long shelf-life which are intended to enable the user to carry out analyses in the daily routine in a simple and reliable manner.

A method for stabilizing aqueous coenzymes using chelating agents and azides is known from JP 84/82398. However, a disadvantage of this method is that it is necessary to add azide which is nowadays classified as cancerogenic and which, moreover, has an inhibitory effect on many enzymes.

It is also known that coenzyme solutions can be stabilized by adding heavy metal salts, for example in the form of copper (II) ions, and thus prevent an increase of the reagent blank (DE 195 43 493 or EP 0 804 610). However, degradation products may form during long storage periods or at high storage temperatures (already above 10°C) which inhibit the dehydrogenase enzyme to be determined and thus result in measured values that are too low. A reagent that can be stabilized over a long period (3 months and more) of constant quality which thus, among other things, does not require repeated calibrations is not available at present.

Hence the object of the present invention is to provide an improved stable liquid reagent containing a coenzyme for hydrogen-transferring enzymes which is suitable for determining dehydrogenase activity or corresponding substrates.

The object is achieved by an aqueous solution which contains a coenzyme for hydrogen-transferring enzymes such as NAD, NADP or an appropriate derivative in an oxidized or reduced form (so-called regenerating systems) and one or several organic compounds or salts derived therefrom with a pKa value between 1.5 and 6.0 and/or a nitrogen compound of the general formula (I)



(I)

in which the residues R^1, R^2 and R^3 are the same or different and denote hydrogen, a saturated or unsaturated alkyl or aryl group. Suitable alkyl groups are in particular those which have one to ten carbon atoms. Furthermore the alkyl groups can be straight-chained or branched. Suitable aryl groups according to the invention are substituted or unsubstituted phenyl groups which are optionally bound via an alkenyl group which can have 1 to 8 carbon atoms. Nitrogen compounds of the said general formula (I) i.e. in particular hydroxylamine derivatives such as hydroxylamine, O- or N-alkylhydroxylamine having one to six carbon atoms or O-benzylhydroxylamine or salts derived therefrom such as sulfates, phosphates or ammonium salts have proven to be particularly suitable according to the invention. In addition suitable hydroxylamine derivatives are characterized by a complexing effect towards the

degradation products of the coenzyme.

In addition the stability of the solutions can be further improved when the solution additionally contains a complexing agent i.e. a ligand which has two or more coordination positions. Bidentate ligands such as ethylene diamine and tetradentate or multidentate ligands such as ethylenediamine-N,N,N,N-tetraacetic acid (EDTA) or appropriate salts thereof, especially the disodium salt, crown ethers or cryptands have proven to be advantageous. This corresponds to a concentration of the complexing agent of about 0.5 to 30 mM, preferably of 1.0 to 5.0 mM.

Organic compounds or salts derived therefrom that are added according to the invention with a pKa value between ca. 1.5 and 6.0 are especially organic acids which have a complexing action and a buffering action in the pH range of 1.0 to 7.0 such as citric acid and water-soluble salts derived therefrom.

The concentrations of the organic compounds, salts or hydroxylamine derivatives that are to be added according to the invention can vary within wide limits i.e. between ca. 0.001 and 1.0 M. A concentration of ca. 5 to 200 mM has proven to be particularly suitable for citric acid or citrate. In numerous cases ca. 50 mM citric acid or citrate already resulted in the desired effect. The preferred concentration range for the hydroxylamine derivative according to the invention that is to be added in addition to or in the absence of an organic compound with a suitable pKa value is between about 2 and 300 mM. The pH value of the stabilized aqueous solution can be between 1.0 and 7.0, a pH value between

ca. 2.0 and 4.0 or of ca. 3.0 having proven to be particularly advantageous.

Moreover, it has proven to be particularly advantageous when the reagent containing NAD or NADP contains a hydroxylamine derivative and optionally additionally a citrate salt and when boric acid or a borate salt is additionally present in an optional further reagent that may be necessary to determine corresponding hydrogen-transferring analytes that contains in particular buffers necessary for the determination such as N-methylglucamine (MEG), substrates and optionally other auxiliary substances. The concentrations set forth above also apply to this special embodiment. Furthermore, ca. 20 to 200 mM have proven to be particularly advantageous for citrate or citric acid and ca. 10 to 150 mM for the respective hydroxylamine derivative. A concentration range of ca. 50 to 200 mM has proven to be particularly suitable for the boric acid derivative which is preferably added to the substrate solution (so-called reagent 1) which does not contain NAD or NADP.

Substances which have a good buffer capacity between ca. pH 8.5 and 10.0 such as the so-called Good buffers (tricine, bicine, TAPS, AMPSO, CHES, CAPSO, AMP, CAPS), carbonates of alkali metal ions, MEG, TRIS and phosphate buffer are basically suitable as buffers for the reagent containing substrate. Mixtures of the said buffer substances have also proven to be suitable for the solution according to the invention. In addition it has proven to be advantageous when the buffer concentration is between ca. 10 and 1000 mM, preferably between 200 and 600 mM. Furthermore, the addition of boric acid or soluble salts and derivatives thereof to the alkaline buffer solution (reagent 1) which primarily determines

the working pH value has proven to be advantageous. The concentration of suitable boric acid components is preferably between about ca. 50 and 200 mM, particularly preferably about 100 mM.

Suitable coenzymes in the sense of the present invention are in particular NAD and NADP, and also modified coenzymes such as thioNAD(P) or NHxDP (=nicotinamide hypoxanthine dinucleotide phosphate). The coenzymes can be present at a concentration of approximately 1.0 to 100 mM in the reaction cuvette; a range of 5.0 to 15.0 mM is preferred.

The stabilized coenzyme solutions according to the invention are preferably used in the form of aqueous solutions. Furthermore the ready-to-use reagent is also stable over a wide time period as a granulate, powder mixture and as a lyophilisate. Thus no signs of reagent decomposition whatsoever are found at temperatures of 2° to 8°C within 15 months. Under stress i.e. at a temperature of ca. 35°C for 2 weeks or treatment at ca. 42°C for five days, it was shown that the solution containing one or several additives according to the invention remained qualitatively unchanged i.e. stable.

A further subject matter of the invention is a method for determining a hydrogen-transferring analyte or a corresponding dehydrogenase in the presence of a hydrogen accepting coenzyme wherein the coenzyme is present in a stabilized aqueous solution as described above.

The determination is carried out in particular in samples of biological origin such as whole blood, serum

or plasma, or other human or animal sources or in plant extracts. The sample can be prepared using physiological saline. In such a case a 0.9 % NaCl solution is advantageously used as a control value.

If it is intended to determine the enzyme activity of a dehydrogenase such as a lactate dehydrogenase, a substrate solution e.g. a lactate solution in a substance (mixture) buffering at ca. pH 9.4 (37°C) is used. In this case the substrate can be used in the usual concentrations known to a person skilled in the art, preferably in a range of 40 to 80 mM.

In order to determine a hydrogen-transferring analyte such as lactate, the respective dehydrogenase, e.g. LDH, is added first in a substance buffering between pH 8.5 and 10.0. Usually a dehydrogenase quantity of approximately 70 to 500 U/l, preferably of 110 to 220 U/l is sufficient. The determination is usually carried out at ca. 37°C.

In addition to lactate which was described as an example, it is also possible to similarly determine glutamate or ammonia, alcohol, glyceraldehyde-3-phosphate, glucose or other parameters that can be converted by a suitable coenzyme-dependent dehydrogenase. This applies in a corresponding manner to the determination of the enzyme activity of such dehydrogenases.

A further subject matter of the invention is a so-called test kit for carrying out the enzyme or analyte determination. The kit is essentially composed of two partial reagents. If it is used to determine the

activity of a dehydrogenase, the first reagent contains a hydrogen-transferring analyte (substrate) in a suitable system buffering between pH 8.5 and 10.0. The second reagent has a coenzyme for hydrogen-transferring enzymes such as NAD or NADP and an organic compound having a pKa value between 1.5 and 6.0 and/or a hydroxylamine derivative according to the invention. The second reagent can additionally contain other auxiliary substances such as heavy metal salts or a complexing agent. This applies correspondingly to the determination of an analyte or substrate such as lactate.

Abbreviations

AMP = 2-amino-2-methyl-1-propanol
AMPSO = 3-[(1,1-dimethyl-2-hydroxyethyl)amino-2-hydroxy-propanesulfonic acid
bicine = N,N-bis[2-hydroxyethyl]glycine
CAPS = 3-[cyclohexylamino]-1-propanesulfonic acid
CAPSO = 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid
CHES = 2-[N-cyclohexylamino]ethanesulfonic acid
MEG = N-methylglucamine
TAPS = N-Tris[hydroxymethyl]methyl-3-aminopropane-sulfonic acid
Tricine = N-Tris[hydroxymethyl]methylglycine
TRIS = 2-amino-2-(hydroxymethyl)-1,3-propanol

The invention is further elucidated by the following examples:

Example 1

Reagent 1: 390 mmol/l N-methylglucamine pH 9.4 (37°C); 60 mmol/l lithium L-lactate.

Reagent 2: 60 mmol/l NAD(P) as a lyophilisate, powder mixture, granulate or aqueous solution.

Incubation temperature: 37 ± 0.1°C; measurement wavelength 340 ± 2 nm; path length 7 mm;

Preincubation: 5 minutes; lag phase: 2 minutes; measurement time: 2 minutes.

Reagent 1 = 250 µl; reagent 2 = 50 µl; sample = 7 µl NaCl solution (0.9 % w/v).

The following determinations were carried out (IFCC: recognised reference for the determination of LDH containing lactate, NAD/NADP and N-methylglucamine, pH 9.4; Eur. J. Clin. Chem. Biochem. vol. 32, p. 639-655 (1994)), Table 1:

Table 1

reagent 1	reagent 2	blank value (BV) unstressed [mA/min]	blank value (BV) 5 days, 42°C [mA/min]	calibrator signal -BV unstressed [mA/min]	calibrator signal -BV 5 days 42°C [mA/min]
IFCC + 100 mmol/l borate	IFCC	1.3	6.3	32.4	31.0 = 95.7 %
IFCC	IFCC + 100 mmol/l citrate pH 3.0	2.1	6.2	35.2	33.4 = 95.4 %
IFCC + 100 mmol/l borate	IFCC + 100 mmol/l citrate pH 3.0	1.0	3.0	30.9	29.4 = 95.1 %
IFCC	IFCC + 50 mmol/l hydroxylamine	0.9	3.2	34.4	33.8 = 98.3 %
IFCC + 100 mmol/l borate	IFCC + 50 mmol/l hydroxylamine	0.7	1.8	31.1	30.5 = 98.0 %
IFCC + 100 mmol/l borate	IFCC + 50 mmol/l hydroxylamine + 100 mmol/l citrate pH 3.0	0.7	1.2	29.8	30.3 = 101.7 %
IFCC (prior art)	IFCC	1.5	11.5	35.7	33.7 = 94.5 %

Result: The inventive formulation containing appropriate additives in the partial reagent 1 and/or partial reagent 2 shows a considerably improved blank value with an almost unchanged calibrator blank value compared to the IFCC reference method especially under stress (5 days, 42°C).

Example 2

The initial solutions described in example 1 and the corresponding procedures were used. Citrate and/or various hydroxylamine derivatives in different concentrations and combinations were added to reagent 2 (table 2).

Table 2

reagent 1	reagent 2	blank value (BV) unstressed [mA/min[	blank value (BV) 5 days, 42°C [mA/min]	calibrator signal -BV unstressed [mA/min]	calibrator signal -BV 5 days 42°C [mA/min]
IFCC = reference	IFCC = reference	0.1	10.7	35.7	32.9 =92.1 %
IFCC	IFCC + 20 mmol/l citrate + 50 mmol/l hydroxylamine sulfate	0.8	2.5	31.6	31.2 =98.7 %
IFCC	IFCC + 20 mmol/l citrate + 50 mmol/l hydroxylamine phosphate	0.6	1.7	30.9	30.2 = 98.1 %
IFCC	IFCC + 20 mmol/l citrate + 50 mmol/l O-benzyl-hydroxylamine	-0.9	-0.6	34.8	33.3 =95.7 %
IFCC	IFCC + 20 mmol/l citrate + 50 mmol/l O-methyl-hydroxylamine	-0.7	0.0	35.2	33.6 =95.5 %
IFCC	IFCC + 20 mmol/l citrate + 50 mmol/l N-methyl-hydroxylamine	4.9	11.1	34.6	34.6 =100.0 %

Result: All compounds and salts added to the inventive reagent 2 resulted in an improved recovery after stress (5 days, 42°C) compared to the IFCC reagent.

Example 3

The recovery of the various isoenzymes was also demonstrated using the formulation according to the invention. This must correspond to the recovery of the recognized IFCC recommendation (table 3).

The determinations were carried out using the IFCC reagent described in example 1 (prior art) compared to a reagent according to the invention.

Table 3

reagent 1	reagent 2	activity iso-enzyme 1 [U/l]	activity iso-enzyme 2 [U/l]	activity iso-enzyme 3 [U/l]	activity iso-enzyme 4 [U/l]	activity iso-enzyme 5 [U/l]
IFCC	IFCC	536	528	735	325	382
IFCC + 100 mmol/l borate	IFCC + 50 mmol/l hydroxylamine + 100 mmol/l citrate pH 3.0	536	523	736	301	368

Result: The recovery of the five LDH isoenzymes was demonstrated with the reagent according to the invention.

If the said modifications were carried out on the formulation, it is possible to provide a liquid LDH reagent which remains stable during storage (> 12 months)

and transport (even at temperatures > 8°C). The resulting advantages for the user are obvious and are shown in the description of the invention.